Necrobiosis Lipoidica: Ultrastructural and Biochemical Demonstration of a Collagen Defect

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Ten patients with necrobiosis lipoidica lesions were studied. Five patients had diabetes mellitus. The age of the patients varied from 15 to 73 years and the duration of the skin lesions was from 2 to 20 years. Histologically, the lesions were characterized by degeneration of collagen and elastin. In some lesions elastin fibers could be seen in areas devoid of normal-looking collagen. Electron microscopy revealed loss of cross-striation of collagen fibrils and a marked variation in the diameter of individual collagen fibrils. The concentration of collagen, measured by assay of hydroxyproline, a collagen-specific amino acid, was markedly decreased in the lesional skin, but the ratio of type I/III collagen was unchanged in the affected skin. Fibroblasts established from affected skin synthesized less collagen than cells derived from healthy-looking skin. The decreased collagen synthesis was due to a decreased amount of messenger RNA for type I procollagen, measured by hybridization with a specific human cDNA clone. The production of collagenase by these fibroblasts was not increased. Our results thus indicate that in necrobiosis lipoidica lesions, collagen fibrils are defective and the amount of collagen is reduced, probably due to decreased synthesis of collagen by affected fibroblasts. *J Invest Dermatol 88:227–232, 1987*

Necrobiosis lipoidica is a chronic skin disease that is often associated with diabetes mellitus. Typical lesions are irregularly demarcated, yellowish lesions on the shins. On histologic examination there is degeneration or necrobiosis of collagen and polymorphic cellular infiltrates composed of lymphoid cells, fibroblasts, and histiocytes [1]. Sometimes, the dermis contains granulomatous foci composed of epitheloid cells and giant cells. The degeneration and hyalinization of collagen bundles adjacent to the granulomas are variable. The basic etiology of necrobiosis lipoidica is unknown. However, focal degeneration of collagen has been suggested to have a central role in its pathogenesis [1]. The purpose of the present study was to examine collagen by ultrastructural and biochemical means in necrobiosis lipoidica lesions.

PATIENTS AND METHODS

The biopsy samples were taken from affected and nonaffected (site-matched) skin of 10 patients. The clinical characterization of the patients given in Table I demonstrates heterogeneity in the ages of the patients and the duration of disease. The age of the patients varied from 15 to 73 years, and duration of necrobiotic lesions from 2 to 20 years. Nine of 10 were females and 5 had diabetes mellitus.

Skin Biopsies Control samples (site-matched) were taken during therapeutic operations on age-matched patients in the Dermatological Clinic, Oulu University Central Hospital, Finland. All the samples were taken in accordance with the Declaration of Helsinki.

Skin samples from necrobiotic lesions of 10 patients were excised for light microscopy, and, in 4 patients, for electron microscopy.

Samples for light microscopy were fixed in 10% phosphate-buffered formalin, processed routinely, and embedded in paraffin. Sections were cut at 5 μm and stained with H & E and Verhoeff-van Gieson stains. Specimens for electron microscopy were fixed in 4% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips 410 LS transmission electron microscope.

Primary cell cultures were established by routine methods, and subcultivated on plastic culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 50 μg/ml of ascorbate, 290 μg/ml L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Analyses of fibroblast cultures were carried out at 4–8 passages of subcultivation.

Collagen Biosynthesis Studies Fibroblasts at confluence were incubated for 24 h in DMEM supplemented as above, except that the serum was replaced with 2% dialyzed fetal calf serum, and [^{14}C]proline (2 μCi/ml) was added. After the labeling period the medium was collected and proteinase inhibitors were added to give final concentrations of 25 mM Na_2EDTA, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, and 1 mM paraaminobenzamidine. The medium proteins were then precipitated by adding ammonium sulfate to a final concentration of

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Abbreviations:
DMEM: Dulbecco's modified Eagle's medium
GGT: galactosylhydroxylysyl glucosyltransferase
PH: prolyl 4-hydroxylase
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Table I. Clinical Data on Patients With Necrobiosis Lipoidica

Code	Sex	Age (years)	Diabetes, Duration (years)	Clinical Findings
1	F	69	Yes, 17	On the anterior site of legs, lesions for 10 years
2	F	73	No	On the left leg, numerous lesions for 8 years
3	F	56	No	On the left leg, some lesions for 3 years
4	F	18	Yes, 6	On the lower legs, lesions for 5 years
5	F	65	No	Above the ankles, numerous lesions for 20 years
6	F	50	Yes, 10	On the legs, lesions for 2 years
7	F	15	Yes, 14	On the lower legs, lesions for 8 years
8	F	49	No	On the legs, lesions for 7 years
9	F	53	No	On right legs, 2 lesions for 5 years
10	M	20	Yes, 13	On legs, some lesions

290 mg/ml, and the precipitates were collected by centrifugation for 30 min at 10,000 *g* after stirring overnight at 4°C. This material was used for the assays of [^{14}C]hydroxyproline and total incorporation of ^{14}C radioactivity by a specific radiochemical method [2].

The cell layer was rinsed with phosphate-buffered saline and the cells scraped with a rubber policeman into 2 ml of 0.4 M NaCl, 0.1 M Tris-HCl, pH 7.5, containing the proteinase inhibitors described above. The cells were sonicated at 60 Hz for 30 s. These samples were used for assays of total cell layer protein [3] and DNA [4].

Part of the ammonium sulfate precipitate of the culture medium was used for 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after reduction [5] and radioactive peptides were visualized by fluorography [6].

Assay of Type I Procollagen mRNA Steady-State Level Total RNA was isolated as described previously [7] and used for dot-blot hybridization assay [8]. The RNA samples were dotted (0.3–0.9 μg of total RNA) onto nitrocellulose paper, and the filters were hybridized with recombinant plasmid Hf677 containing cDNA for human proα1(I) collagen mRNA [9]. The recombinant plasmid was labeled with [^{32}P]nucleotides to a specific activity of 5–8 × 10^8 cpm/μg by nick-translation [10]. The amount of recombinant ^{32}P-labeled plasmid hybridized to mRNA was visualized by autoradiography using Kodak X-Omat film and cassettes with intensifying screens. The autoradiograms were quantitated by scanning with a Kontes K 495000 densitometer connected to a Spectra-Physics SP4100 computing integrator.

Assays of Enzyme Activities The cells were grown to early confluence, harvested by trypsinization, and stored in the form of a pellet at −70°C for up to 3 weeks. After thawing, they were homogenized with a tight Teflon-glass homogenizer (1200 rpm, 50 strokes) in a cold solution containing 0.2 M NaCl, 0.1 M glycine, 0.1% (wt/vol) Triton X-100, 0.01% (wt/vol) soya-bean trypsin inhibitor, and 0.02 M Tris-HCl buffer, pH 7.5 (3–4 × 10^6 cells/ml). The homogenates were centrifuged at 15,000 *g* for 30 min at 4°C and aliquots of the supernatants were taken for the enzyme assays.

Skin samples were homogenized with a Polytron tissue homogenizer in the solution described above. The homogenates were centrifuged at 15,000 *g* for 30 min, and aliquots of the supernatant were used for protein and enzyme assays.

Prolyl 4-hydroxylase (PH) activity was assayed by measuring the formation of radioactive 4-hydroxyproline using a [^{14}C]proline-labeled type I protocollagen substrate [11].

Galactosylhydroxylysyl glucosyltransferase (GGT) activity was assayed by determining the radioactive glucosylgalactosylhydroxylysine formed in a gelatinized calf skin collagen substrate [11].

For assay of collagenase activity, fibroblasts were cultured in serum-free DMEM for 6 h. The medium was collected and aliquots were subjected to brief trypsin proteolysis using 0.1–10 μg trypsin per ml for 10 min at 25°C. Trypsin was then inactivated by adding soya-bean trypsin inhibitor (50 μg/ml), and collagenase activity was assayed by incubating samples with radioactive type I collagen, as described previously [12]. The collagenase activity was expressed as degradation of ^{3}H-labeled collagen, dpm × h^{-1}/mg DNA.

Analyses of Total Collagen and Collagen Types The amount of hydroxyproline, a measure of collagen, was determined by a specific colorimetric assay [13].

For determination of genetically distinct collagen types, tissue specimens were homogenized in 0.5 M acetic acid and submitted to limited proteolysis by pepsin (Worthington, 2 × crystallized), at a final concentration of 300 μg pepsin per ml. The samples were incubated for 3 h at 24°C, followed by 16 h at 4°C. The pepsin-solubilized material was recovered by centrifugation for 60 min at 37,000 *g* at 4°C, and the insoluble material was subjected to further pepsinization as above. The supernatants containing the pepsin-solubilized material were combined, and protease inhibitors at the concentrations indicated above were added. The pH of the samples was adjusted to 8.5 by adding 1 M Tris, and the samples were incubated for 60 min at 4°C to inactivate pepsin. The samples were then dialyzed against 0.4 M NaCl, 10 mM Tris-HCl, pH 7.5, containing the proteinase inhibitors. Collagen was precipitated by adding NaCl to a final concentration of 4.4 M, and the precipitate was collected by centrifugation.

The SDS-PAGE was performed using 8% polyacrylamide gels, with and without delayed reduction with 2-mercaptoethanol [14]. The collagen polypeptides were visualized by staining with Coomassie Brilliant Blue and quantitated with an automatic computing densitometer.

Assay of DNA Synthesis The DNA synthesis activity was estimated by determining the incorporation of [^{3}H]thymidine into the trichloroacetic acid-precipitable material. The cells were transferred to a 24-well plate, approximately 2 × 10^4 cells per well. After 48-h preincubation the cells were labeled with [^{3}H]thymidine (1 μCi/well) for 4 h. The cells were then washed thoroughly with phosphate-buffered saline and disrupted by incubating for 1 h at 40°C in 1% (wt/vol) SDS, 1 mM Na_2EDTA, and 50 mM Tris-HCl, pH 7.5, supplemented with 65 μg/ml of proteinase K. The lysates were then precipitated with 1 vol of 20% (wt/vol) trichloroacetic acid containing 100 μg of calf thymus DNA as a carrier. The precipitates were collected by centrifugation at 15,000 *g* for 20 min, washed twice with 10% (wt/vol) trichloroacetic acid, dissolved in Lumagel, and the radioactivity counted.

For statistical analyses Student's *t*-test was used.

RESULTS

Light Microscopy Findings The samples from the clinically necrobiotic skin showed collagen degeneration and necrosis at various levels of the dermis. The necrotic foci were surrounded by mononuclear cell infiltrates, histiocytic cells, lymphocytes, and plasma cells. Histiocytic cell granulomas were seen in 6 of 10 cases (Fig 1). The elastica stain, Verhoeff-van Gieson, revealed the elastic fibers of degenerated and necrotic areas to be short and

Figure 1. Light microscopic picture from necrobiosis lipoidica skin. The collagen and elastin bundles are regular in noninvolved areas but are lost in areas of degeneration and of giant and epitheloid cell granulomas. Some granulomas show traces of elastin (*arrow*). Verhoeff-van Gieson. ×140.

uneven in thickness, and disorientated, few in number, or totally lost. As a rule, the granulomas were totally devoid of collagen and elastic fibers, but some granulomas contained traces of elastic fibers (Fig 1).

Electron Microscopy Findings Collagen degeneration and necrosis appeared as swollen or frazzled fibers with disappearing periodic banding (Fig 2), and as loose filamentous and amorphous, slightly granular material in more advanced cases. The elastic fibers were seen to be thick masses of homogenous elastica with central skeleton fibrils and peripheral elastic-fiber microfibrils (not shown). The granulomas were composed of histiocytic and fibroblastic cells and sometimes of multinucleated histiocytic cells. Some granulomas were in the vicinity of the capillaries. Both histiocytes and fibroblasts showed degenerative changes, cytoplasmic peripheral villi, swellings, dividing lines in the cytoplasm, and pinching off of parts of the cytoplasm (Fig 3). Lysosomal dense bodies and other phagocytized material were also noticed, as well as a prominent rough endoplasmic reticulum in some fibroblasts.

Skin Biopsy Studies The concentration of hydroxyproline, a measure of collagen, was markedly decreased in the skin biopsy specimens taken from the affected skin of the patients (Fig 4). The mean value was 9.4 ± 3.7 (SD) μg/mg wet weight in affected skin, and 15.9 ± 11.9 μg/mg in the nonaffected skin ($p < 0.05$). The concentration of DNA, reflecting tissue cellularity, and the activities of PH ($p < 0.01$) and GGT ($p < 0.01$) were increased in the affected skin compared to the samples taken from nonaffected skin or from healthy controls (Table II).

The ratios of types I and III collagen were estimated from skin biopsies after limited pepsin proteolysis and 4.4 M NaCl precipitation. The mean proportion of type III collagen in the biopsy specimens from the affected skin of 3 patients (nos. 5, 6, and 9) was 23.1 ± 5.6% (SD), and in control samples 18.4 ± 8.1%.

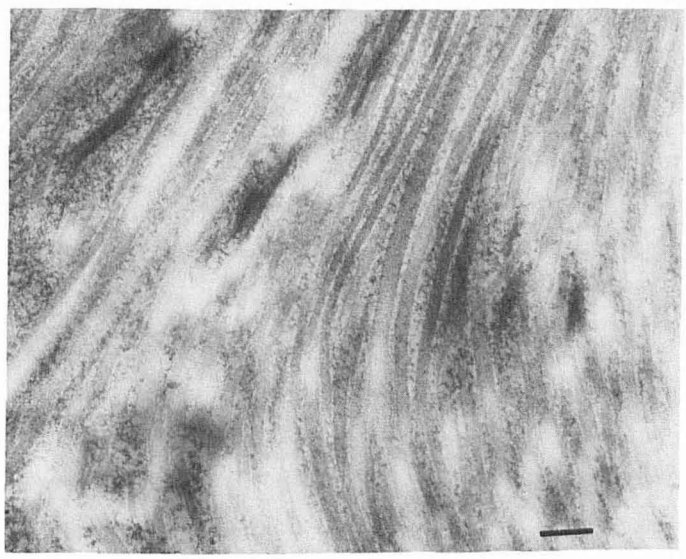

Figure 2. Electron microscopic picture from necrobiotic skin. The degenerated collagen fibers show granularity, indistinct borders, and loss of periodic banding. *Bar* = 0.25 μm.

The solubility of collagen in 0.5 N acetic acid was also measured. It is known that the solubility of collagen is increased in situations with increased synthesis of collagen [15]. In affected skin, 2.1 ± 1.3% (SD) and in control skin, 2.6 ± 0.3% of the collagen was acid-soluble.

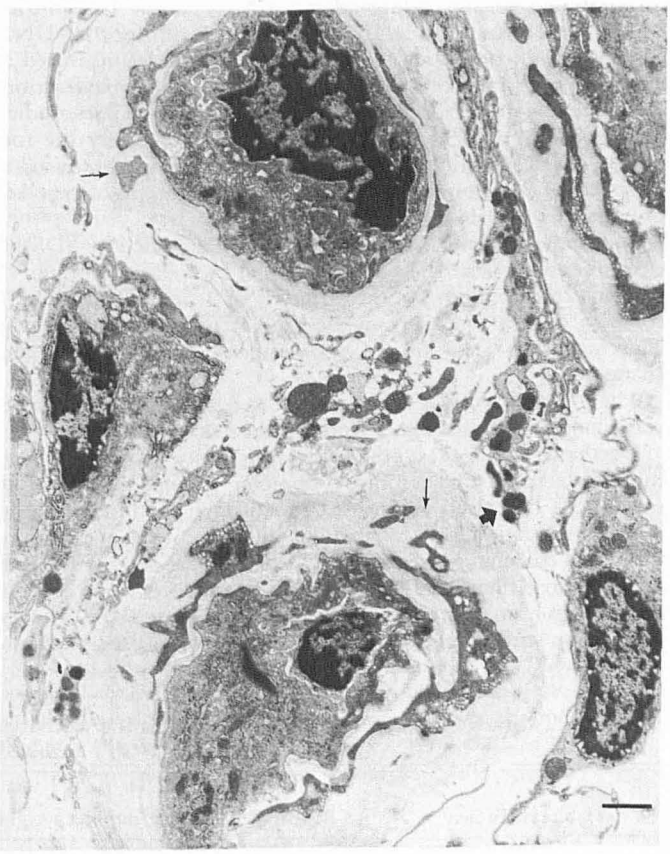

Figure 3. Electron micrograph of epitheloid cell granuloma. The histiocytic and fibroblastic cells are degenerating and show peripheral cytoplasmic villi, i.e., pinching off of the peripheral cytoplasm (*thin arrows*). A histiocytic cell with far-advanced degeneration and lysis contains dense lysosomal bodies (*thick arrow*). *Bar* = 1.0 μm.

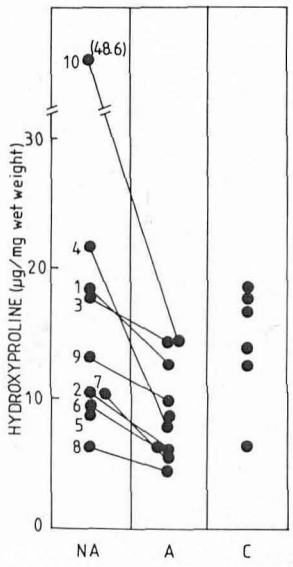

Figure 4. Concentration of hydroxyproline in the nonaffected (*NA*) and affected (*A*) skin of patients with necrobiosis lipoidica and in the controls (*C*). Hydroxyproline was assayed as described in *Patients and Methods*. The *numbers* beside the symbols refer to the subjects in Table I.

Fibroblast Culture Studies To examine the mechanisms behind the decreased concentrations of collagen in the skin of necrobiosis lipoidica patients, fibroblast cultures were established from affected and nonaffected skin. Radioactive thymidine incorporation, reflecting cell proliferation, was 3.04×10^3 dpm/μg DNA in cells from affected skin and 2.43×10^3 dpm/μg DNA in healthy skin fibroblasts. Total protein synthesis, measured as the incorporation of [^{14}C]proline into the nondialyzable fraction, was markedly decreased in 3 necrobiosis lipoidica cell lines studied (patients 1, 3, and 5). Collagen synthesis, assayed by the formation of [^{14}C]hydroxyproline, was also significantly reduced in these same cell cultures (Table III). The relative ratio of type I to III collagens was unchanged (not shown).

Reduced collagen synthesis was also demonstrated by SDS-gel electrophoresis. There was a decrease in the relative amount of proteins corresponding to the positions of proα1(I) and proα2(I) collagen chains, especially in the cells of patient no. 3 (Fig 5). The molecular sizes of the procollagen molecules were unchanged.

The PH activity was slightly decreased and the GGT activity was unaltered in fibroblasts from affected skin (Table III).

Decreased collagen synthesis by fibroblasts established from the affected skin of necrobiosis lipoidica patients could be due either to a decreased amount of procollagen mRNA or to decreased translational efficiency. To study the mechanism behind decreased collagen synthesis, cellular RNA was dot-blotted onto nitrocellulose filters and hybridized with a cDNA clone complementary to human proα1(I) collagen mRNA sequences. The hybridization studies revealed a marked decrease in type I collagen mRNA in the fibroblasts derived from the affected skin of the 2 patients (nos. 3 and 5) studied (Fig 6). The amount of type I collagen mRNA was 50% in cell line no. 3 and 57% in cell line no. 5 compared with the corresponding value in cells derived from the healthy skin of the same patients.

Since increased degradation of collagen by collagenase might contribute to the reduced amount of collagen in necrobiosis lipoidica lesions, collagenase activity was determined from the media of cell cultures after brief trypsin activation. The results indicated that in the 3 cell lines analyzed, the mean activity of collagenase was 14.3×10^6 dpm/h/mg DNA in cell cultures established from healthy skin and 12.0×10^6 dpm/h/mg DNA in fibroblasts derived from affected skin.

DISCUSSION

In the present study we demonstrated that large areas of skin lesions in patients with necrobiosis lipoidica were devoid of normal-looking collagen. In electron microscopy studies, the disappearance of regular cross-striations of collagen fibrils was the most prominent finding. Variation in the diameter of collagen fibrils was also noted in the lesional skin. The reason for this is unknown. It is possible that at early stages of the degenerative process, the regular structures of collagen fibrils are destroyed, leading to abnormal-looking fibrils. Another possibility could be that during regeneration, newly synthesized collagen molecules are unable to form structurally normal collagen fibrils. Elastin, another major component of dermal connective tissue, was also decreased in the affected skin. However, there were clearly detectable areas where collagen had totally disappeared, and some elastin fibers were still to be seen. This may reflect the fact that elastin is highly resistant to the attack of proteolytic enzymes [16]. Since various inflammatory cells produce elastase-like enzymes [17], the decrease of elastin in most of the lesions is plausible.

In the biochemical studies, the concentration of hydroxyproline was markedly reduced in skin specimens obtained from the lesional skin. The concentration of hydroxyproline in the nonaffected skin was within the range of healthy controls, which indicates that the deficiency of collagen was limited to the lesional skin of the patients. The concentration of DNA and the activities of PH and GGT were increased in the affected skin. The increased DNA concentration could have been due to the accumulation of cellular infiltrate, which was obvious on histologic examination. The elevated number of inflammatory cells could also have contributed to the increased activities of PH and GGT, since it is known that PH and GGT can be found in inflammatory cells [18], and PH and GGT activities have been found to be increased, e.g., in sarcoidosis and lichen planus lesions [19,20]. Constant degeneration and regeneration of collagen may also induce the synthetic activity of fibroblasts in vivo, leading to increased levels of PH and GGT.

In order to study the mechanisms behind the reduced concentration of collagen in necrobiosis lipoidica, fibroblast cultures were established and studied in detail. The fibroblasts derived from the lesional skin clearly showed a decreased capacity to synthesize collagen in vitro. The reason for this is currently unknown. However, it is possible that cells were affected in vivo,

Table II. Concentration of DNA and the Activities of Prolyl 4-Hydroxylase (PH) and Galactosylhydroxylysyl Glucosyltransferase (GGT) in the Skin of Patients With Necrobiosis Lipoidica[a]

Site of Biopsy	No. of Patients	DNA (μg/mg wet weight)	PH (dpm $\times 10^{-3}$/mg protein)	GGT (dpm $\times 10^{-3}$/mg protein)
Patients				
Nonaffected skin	10	2.41 ± 0.78	7.49 ± 3.48	6.06 ± 4.07
Lesional skin	10	4.06 ± 2.81	15.59 ± 6.14[b]	11.54 ± 4.75[b]
Control subjects	6	2.04 ± 0.56	6.53 ± 2.20	6.26 ± 1.53

[a]The biochemical analyses were performed as described in *Patients and Methods* and the values are the mean ± SD.
[b]Significantly different from nonaffected skin; $p < 0.01$.

Table III. Collagen Production and the Activities of Prolyl 4-Hydroxylase (PH) and Galactosylhydroxylysyl Glucosyltransferase (GGT) in Necrobiosis Lipoidica Fibroblasts in Culture

Cell Line (code)	Total Incorporation[a] (dpm × 10^{-3}/mg DNA)	[^{14}C]Hydroxyproline[a] (dpm × 10^{-3}/mg DNA)	PH[b] (dpm/μg protein)	GGT[b] (dpm/μg protein)
Necrobiosis lipoidica				
Nonaffected (1)	9.42	2.01	105.7	39.1
Nonaffected (3)	8.89	2.00	170.1	45.1
Nonaffected (5)	7.88	1.85	179.5	40.0
Mean ± SD	8.73 ± 0.78	1.95 ± 0.09	151.6 ± 40.5	41.4 ± 3.2
Lesion (1)	3.19	0.70	88.1	57.8
Lesion (3)	1.10	0.16	98.1	43.6
Lesion (5)	1.87	0.43	131.3	49.7
Mean ± SD	2.05 ± 1.06[c]	0.43 ± 0.27[c]	105.8 ± 22.6	50.4 ± 7.1
Controls (n = 5)	12.9 ± 3.8	3.00 ± 0.46	185.7 ± 42.6	49.5 ± 8.0

[a]Fibroblast cultures from necrobiosis lipoidica patients and normal human subjects were labeled with [^{14}C]proline for 24 h; [^{14}C]hydroxyproline and total radioactivity were then assayed from the culture medium as described in *Patients and Methods*.
[b]Fibroblasts at early confluency were harvested and used for the assay of PH and GGT, as described in *Patients and Methods*.
[c]Significantly different from nonaffected skin; $p < 0.01$.

i.e., by mediators of inflammatory cells [21–24], and were unable to produce normal amounts of collagen in vitro. The reduced synthesis of collagen was found to be due to a decreased amount of collagen mRNA, indicating that collagen synthesis was affected at the pretranslational level. This is in agreement with previous studies in keloid cells [25], scleroderma fibroblasts [26], and virus-transformed cells [27], in which the rate of collagen biosynthesis correlated well with the abundance of collagen mRNA.

The reduced amount of collagen could also be due to increased degradation of collagen in vivo. Various metalloproteinases from inflammatory cells and fibroblasts could participate in the degradation of collagen in necrobiosis lipoidica [28]. The production of collagenase by fibroblasts was not increased, however, in the present study, indicating that fibroblast collagenase may not have a central role in the degradative process. It thus seems that in the development of necrobiosis lipoidica lesions, the accumulation of inflammatory cells leads to degeneration of the connective tissue matrix. In vivo there is still some regenerative process, which is reflected in increased levels of enzymes of collagen biosynthesis. Future studies on the interactions of connective tissue, inflammatory cells, and various mediators could further elucidate the mechanistic details behind the reduced connective tissue in necrobiosis lipoidica [29].

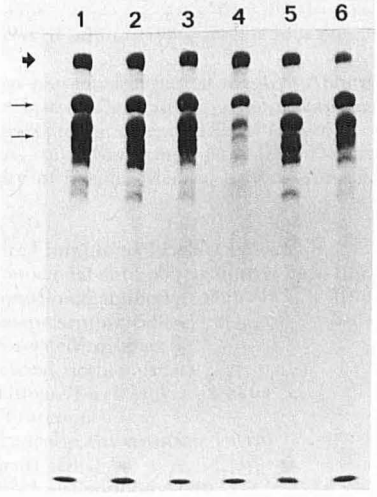

Figure 5. The analysis of medium proteins of cell cultures from necrobiosis lipoidica patients by 6% SDS-PAGE. The cells were labeled with [^{14}C]proline, the medium proteins were precipitated with ammonium sulfate, and analyzed (10,000 dpm/well) by electrophoresis after reduction. The *thick arrow* indicates the migration position of fibronectin, and the *thin arrows* the positions of proα1(I) and proα2(I) chains. *Lanes 1 and 2* represent samples from patient no. 1; *lanes 3 and 4* from patient no. 3; and *lanes 5 and 6* from patient no. 5. *Lanes 1, 3, and 5* are from nonaffected skin and *lanes 2, 4, and 6* from affected skin.

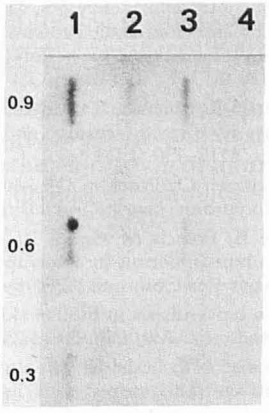

Figure 6. Determination of type I procollagen mRNA abundance in necrobiosis lipoidica fibroblasts. Total RNA was isolated and dotted on nitrocellulose filters (0.3, 0.6, and 0.9 μg/well). RNA was hybridized with ^{32}P-labeled human proα1(I) collagen cDNA probe. The RNA-[^{32}P]DNA hybrids were visualized by autoradiography and quantitated by scanning the bands with a densitometer. *Lanes 1 and 2* represent assay of the cell line from patient no. 3, and *lanes 3 and 4* that from patient no. 5. *Lanes 1 and 3* are from nonaffected skin and *lanes 2 and 4* from affected skin of the patients.

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